OLIGONUCLEOTIDE FOR DETECTING SALMONELLA AND
METHOD OF DETECTING SALMONELLA

Field of the Invention

The present invention relates to oligonucleotides for detecting mRNA (hereinafter, sometimes referred to as "target RNA") of toxin genes invA or stn of Salmonella bacteria, that is commonly known as cause of bacterial food poisoning, and a detection method using the same.

Prior Art

Detection and identification of Salmonella bacteria in clinical examinations, public health examinations, food evaluations and food poisoning examinations have conventionally conducted via cultivation of Salmonella bacteria on a medium to which food or the patient's feces are directly applied or cultivating in a growth medium, followed by cultivation in a selective medium.

Such a culturing method lacks speed because it requires at least 18 hours of cultivation time. Recently, detection methods such as gene amplification methods including PCR methods have been developed to accomplish rapid detection. However, when DNA is the subject to be detected, there is a possibility that a positive result may result from amplification of DNA derived from a killed bacteria contained in the pasteurized food or the like. Moreover, in a detection by electrophoresis following amplification, commonly carried out in the PCR method, there is a possibility that a negative sample will be contaminated due to scattering of amplified products, which may lead to a false positive result.

Due to the fact that RNA is rarely present in killed bacteria, RNA is also detected by a PCR method wherein RNA is preconverted to DNA by a reverse transcription reaction (RT-PCR method). However, since the originally existing DNA is amplified together with RNA, DNA of the

killed bacteria is amplified as mentioned above. As a result, there is a possibility that the results are erroneously judged as positive. Removal of the originally existing DNA is required to avoid the false judgment. The detection procedure thus becomes complicated and cannot be conducted rapidly.

NASBA, 3SR, and the like that amplify specific RNA sequences with reverse transcriptase and RNA polymerase are known (see, for example, Japanese Patent Publication No. 2,650,159, with regard to the NASBA method, and European Patent Publication No. 373,960, with regard to the 3SR method). In these methods, the following procedures are carried out: using a specific sequence as a template, a double-stranded DNA including a promoter sequence is synthesized with a primer containing the promoter sequence, reverse transcriptase and Ribonuclease H; this double-stranded DNA is used as a template in synthesizing an RNA containing the specific sequence with an RNA polymerase; and, subsequently, this RNA provides a template in a chain reaction for synthesizing a double-stranded DNA containing the promoter sequence. NASBA, 3SR, and the like allow amplification of only a specific sequence at a constant temperature; moreover, since they allow amplification at a constant temperature, they are considered suitable for automation.

Because the RNA amplification methods such as NASBA and 3SR methods involve relatively low temperature reactions (41°C, for example), the target RNA may form an intramolecular structure that inhibits binding of the primer, which may reduce the reaction efficiency. Therefore, they require subjecting the target RNA to heat denaturation (heat denaturation at 65°C, for example) prior to the amplification reaction so as to destroy the intramolecular structure thereof and thus to improve the primer binding efficiency. As a result, the simplicity and speed of the methods are impaired. Moreover, when the electrophoresis method is to be employed in the

detection subsequent to the amplification reactions, the problem of false positive results due to scattering of the amplified product arises, as explained above.

Thus, an object of the present invention is to provide an oligonucleotide capable of complementarily binding to an intramolecular structure-free region of Salmonella toxin gene mRNA. That is, an object of the present invention is to provide an oligonucleotide capable of binding to the intramolecular structure-free region and used for amplifying and detecting Salmonella toxin gene mRNA and also to provide a detection method used for simple, rapid and highly sensitive clinical examinations, food examinations, food poisoning examinations, and the like, conducted by amplifying the specific sequence of the target RNA with such an oligonucleotide.

The invention according to claim 1 and intended to accomplish the objects relates to an oligonucleotide for detection of Salmonella toxin gene invA mRNA, which oligonucleotide is capable of specifically binding to Salmonella gene invA mRNA, and comprises at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 1 to 12.

Moreover, the invention according to claim 2 and intended to accomplish the objects relates to an oligonucleotide for detection of Salmonella toxin gene stn mRNA, which oligonucleotide is capable of specifically binding to Salmonella toxin gene stn mRNA, and comprises at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 13 to 18.

Furthermore, the invention according to claim 3 and intended to accomplish the objects relates to a process of amplifying Salmonella gene invA mRNA, wherein a specific sequence of Salmonella gene invA mRNA present in a sample is used as a template for synthesis of a cDNA employing an RNA-dependent DNA polymerase, the RNA of the formed RNA/DNA hybrid is digested by Ribonuclease H to

produce a single-stranded DNA, the single-stranded DNA is then used as a template for production of a double-stranded DNA having a promoter sequence capable of transcribing RNA comprising the specific sequence or the sequence complementary to the specific sequence employing a DNA-dependent DNA polymerase, the double-stranded DNA produces an RNA transcription product in the presence of an RNA polymerase, and the RNA transcription product is then used as a template for cDNA synthesis employing the RNA-dependent DNA polymerase, the amplification process being characterized by employing a first oligonucleotide capable of specifically binding to Salmonella gene invA mRNA and comprising at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 1 to 12 and a second oligonucleotide comprising at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 19 to 23 and having a sequence homologous to a portion of the Salmonella gene invA mRNA sequence to be amplified, where either the first or second oligonucleotide includes the RNA polymerase promoter sequence at the 5' end.

Still furthermore, the invention according to claim 4 and intended to accomplish the objects relates to a process of amplifying Salmonella gene stn mRNA, wherein a specific sequence of Salmonella gene stn mRNA present in a sample is used as a template for synthesis of a cDNA employing an RNA-dependent DNA polymerase, the RNA of the formed RNA/DNA hybrid is digested by Ribonuclease H to produce a single-stranded DNA, the single-stranded DNA is then used as a template for production of a double-stranded DNA having a promoter sequence capable of transcribing RNA comprising the specific sequence or the sequence complementary to the specific sequence employing a DNA-dependent DNA polymerase, the double-stranded DNA produces an RNA transcription product in the presence of an RNA polymerase, and the RNA transcription product is then used as a template for cDNA synthesis employing the RNA-dependent DNA polymerase, the amplification process

being characterized by employing a first oligonucleotide
capable of specifically binding to Salmonella gene stn
mRNA, and comprising at least 10 contiguous bases of any
of the sequences listed as SEQ. ID. Nos. 13 to 18 and a
second oligonucleotide comprising at least 10 contiguous
bases of any of the sequences listed as SEQ. ID. Nos. 24
to 27 and having a sequence homologous to a portion of
the Salmonella gene stn mRNA sequence to be amplified,
where either the first or second oligonucleotide includes
the RNA polymerase promoter sequence at the 5' end.

The invention according to claim 5 relates to a
detection method comprising carrying out the
amplification process according to claim 3 or 4 in the
presence of an oligonucleotide probe capable of
specifically binding to the RNA transcription product
resulting from the amplification and labeled with an
intercalator fluorescent pigment, and measuring changes
in the fluorescent properties of the reaction solution,
with the proviso that the labeled oligonucleotide has a
sequence different from those of the first
oligonucleotide and the second oligonucleotide. The
invention according to claim 6 relates to the detection
method according to claim 5, characterized in that the
probe is designed so as to complementarily bind to at
least a portion of the sequence of the RNA transcription
product, and the fluorescent property changes relative to
that of a situation where a complex formation is absent.
The invention according to claim 7 relates to the
detection method according to claim 6, characterized in
that the probe for detecting the invA mRNA comprises at
least 10 contiguous bases of SEQ. ID. No. 28 or its
complementary sequence. The invention according to claim
8 relates to the detection method according to claim 6,
characterized in that the probe for detecting the stn
mRNA comprises at least 10 contiguous bases of SEQ. ID.
No. 29 or its complementary sequence. The present
invention will be explained below.

An oligonucleotide of the invention capable of specifically binding to Salmonella toxin gene invA mRNA and comprising at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 1 to 12 and an oligonucleotide of the invention capable of specifically binding to Salmonella toxin gene stn mRNA and comprising at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 13 to 18 are characterized by being each capable of specifically binding to a portion that has no steric structure in the target RNA at a relatively low and constant temperature (35 to 50°C). As a result, the oligonucleotides of the present invention are, for example, useful as primers for practicing a nucleic acid amplifying process such as NASBA and 3SR for the target RNA. In addition, because the oligonucleotides of the present invention are capable of specifically binding to the target RNA at a relatively low and constant temperature (35 to 50°C), they can be used for the process of amplifying the target RNA by NASBA or 3SR that is carried out to practice the amplifying process in the above temperature range and at a constant temperature (41°C, for example). The use of the oligonucleotides achieves the effect of making unnecessary the denaturation of RNA prior to the practice of such an amplification process as mentioned above.

The present invention also provides a nucleic acid amplification process for amplifying a specific sequence of the target RNA and a method for detecting an RNA transcription product formed in the nucleic acid amplification process. For example, in the amplification process of the NASBA method, a specific sequence of RNA present in a sample is used as a template for synthesis of a cDNA employing an RNA-dependent DNA polymerase, the RNA of the RNA/DNA hybrid is digested by Ribonuclease H to produce a single-stranded DNA, the single-stranded DNA is then used as a template for production of a double-stranded DNA having a promoter sequence capable of

transcribing RNA comprising the specific sequence or the
sequence complementary to the specific sequence employing
a DNA-dependent DNA polymerase, the double-stranded DNA
produces an RNA transcription product in the presence of
5   an RNA polymerase, and the RNA transcription product is
then used as a template for cDNA synthesis employing the
RNA-dependent DNA polymerase. The process of amplifying
Salmonella toxin gene invA mRNA provided by the present
invention is characterized by employing a first
10   oligonucleotide capable of specifically binding to
Salmonella gene invA mRNA and comprising at least 10
contiguous bases of any of the sequences listed as SEQ.
ID. Nos. 1 to 12 and a second oligonucleotide comprising
at least 10 contiguous bases of any of the sequences
15   listed as SEQ. ID. Nos. 19 to 23 and having a sequence
homologous to a portion of the mRNA sequence to be
amplified, where either the first or second
oligonucleotide includes the RNA polymerase promoter
sequence at the 5' end.
20   Furthermore, the process of amplifying Salmonella
toxin gene stn mRNA provided by the present invention is
characterized by employing a first oligonucleotide
capable of specifically binding to the mRNA and
comprising at least 10 contiguous bases of any of the
25   sequences listed as SEQ. ID. Nos. 13 to 18 and a second
oligonucleotide comprising at least 10 contiguous bases
of any of the sequences listed as SEQ. ID. Nos. 24 to 27
and having a sequence homologous to a portion of the mRNA
sequence to be amplified, where either the first or
30   second oligonucleotide includes the RNA polymerase
promoter sequence at the 5' end.
         There are no particular restrictions on the RNA-
dependent DNA polymerase, the DNA-dependent DNA
polymerase and the ribonuclease H to be used in the
35   amplification process of the present invention. For
example, two or three types of enzymes each having the
relevant activity may be used. However, AMV reverse

transcriptase that has all of these types of activity is most preferably used. Moreover, although the RNA polymerase to be used in the amplification process of the present invention is not particularly restricted, T7

5   phage RNA polymerase or SP6 phage RNA polymerase is preferably used.

In the amplification process of the present invention, an oligonucleotide that is complementary to the region adjacent to and overlapping with the 5' end

10   region of the specific sequence (bases 1 to 10) of the target RNA sequence is added, and the target RNA is cleaved with Ribonuclease H at the 5' end region of the specific sequence to give the initial template for nucleic acid amplification, thereby allowing

15   amplification of RNA even when the specific sequence is not positioned at the 5' end. The oligonucleotide used for the cleaving may, for example, be any of those of SEQ. ID. Nos. 1 to 12 for Salmonella toxin gene invA mRNA, and SEQ. ID. Nos. 13 to 18 for Salmonella toxin

20   gene stn mRNA (provided that it differs from the one used as the first oligonucleotide in the amplification process). In addition, the oligonucleotide for cleaving is preferably chemically modified (for example, aminated) at the 3' hydroxyl group in order to prevent an extension

25   reaction from the 3' end.

The detection method provided by the present invention is characterized by carrying out the amplification process as explained above in the presence of an oligonucleotide probe labeled with an intercalator

30   fluorescent pigment, and measuring changes in the fluorescent properties of the reaction solution. Examples of the oligonucleotide probe include one in which the intercalator fluorescent pigment is bonded to a phosphorus atom in the oligonucleotide through a linker.

35   The probe is characterized in that when it forms a double-stranded chain with the amplification product, separation analysis is not required because the

intercalator portion intercalates into the double-
stranded chain portion to vary the fluorescent
characteristics (Ishiguro, T. et al. (1996), Nucleic
Acids Res. 24 (24) 4992-4997).

5   The probe sequence is not critical so long as it has
a sequence complementary to at least a portion of the
amplification product. However, the probe sequence is
preferably one comprising at least 10 contiguous bases of
the sequence listed as SEQ. ID. No. 28 for Salmonella
10   toxin gene invA mRNA, and one comprising at least 10
contiguous bases of the sequence listed as SEQ. ID. No.
29 for Salmonella toxin gene stn mRNA. Moreover,
chemical modification (for example, glycolic acid
addition) at the 3' end hydroxyl group of the probe is
15   preferred in order to inhibit an extension reaction based
on the probe used as a primer.

          It becomes possible to amplify and detect RNA
comprising a specific sequence of Salmonella toxin gene
invA and stn mRNA or a sequence complimentary to the
20   specific sequence thereof in a single tube at a constant
temperature and in a single step by carrying out the
amplification process in the presence of the probe, as
explained above, and, thus, the amplification process is
easily automated.

25   Brief Description of the Drawings

          Fig. 1 is an electrophoresis photograph (black and
white, negative) of a urea-modified 6% PAGE for samples
prepared by conducting the cleavage experiment of Example
1 on Salmonella toxin gene invA mRNA standard at 41°C
30   using oligo-1 to oligo-12 and RNase H. Lane Nos. 1 to 12
correspond to oligo-1 to -12, respectively. M indicates
markers. Arrows indicate bands showing specific
cleavages.

          Fig. 2 is an electrophoresis photograph (4% agarose
35   gel) for samples prepared from the RNA amplification
reaction of Salmonella toxin gene invA mRNA standard in
Example 2, with an initial RNA amount of 10^4 copies/30

μl, using oligonucleotide probes combinations (a) to (h) listed in Table 2. Lanes 1, 2 show the results for combination a); lanes 3, 4 are for combination b); lanes 5, 6 are for combination c); lanes 7, 8 are for combination d); lanes 9, 10 are for combination e); lanes 11, 12 are for combination f); lanes 13, 14 are for combination g); and lanes 15, 16 are for combination h), respectively, wherein lanes 2, 4, 6, 8, 10, 12, 14 and 16 are controls (diluent alone is used in place of the RNA samples.) Arrows indicate specific amplified bands. A characteristic band could be confirmed for any of the combinations.

Fig. 3 shows the results obtained in Example 3 for samples prepared from the Salmonella invA mRNA standard with an initial RNA amount between 10^1 copies/30 μl and 10^5 copies/30 μl. Upper panel (a) is a fluorescence profile exhibiting the fluorescence increase ratio that increases with the reaction time-course formation of RNA. Lower panel (b) is a calibration curve exhibiting the relationship between the logarithm of the initial RNA amount and the detection time (time at which the relative fluorescence reaches 1.2). It was demonstrated that RNA with initial copies of 10^2 copies/30 μl can be detected by a reaction for about 13 minutes, and that there is a correlation between the initial RNA amount and the detection time.

Fig. 4 is an electrophoresis photograph (black and white, negative) of a urea-modified 6% PAGE for samples prepared by conducting the cleavage experiment of Example 4 on Salmonella toxin gene stn mRNA standard at 41°C using oligo-13 to oligo-18 and RNase H. Lane Nos. 13 to 18 correspond to oligo-13 to -18, respectively. M indicates markers. Arrows indicate bands showing specific cleavages.

Fig. 5 shows the results obtained in Example 5 using samples prepared from the Salmonella stn mRNA standard

with an initial RNA amount of 10^4 copies/30 μl,
exhibiting the fluorescence increase ratio that increases
with the reaction time-course formation of RNA. RNA in
an amount of 10^4 copies/30 μl could be detected by a
reaction for about 11 to 15 minutes for any of the
combinations of primers. ▲: Fluorescence monitoring
employing combination (i) (sample: 10^4 copies); △:
Fluorescence monitoring employing combination (i)
(control); ●: Fluorescence monitoring employing
combination (j) (sample: 10^4 copies); O: Fluorescence
monitoring employing combination (j) (control); ◆:
Fluorescence monitoring employing combination (k)
(sample: 10^4 copies); ◇: Fluorescence monitoring
employing combination (k) (control); ■: Fluorescence
monitoring employing combination (l) (sample: 10^4
copies); and □: Fluorescence monitoring employing
combination (l) (control).

Examples

The present invention will now be explained in
greater detail by way of examples, with the understanding
that the invention is not limited by the examples.

Example 1

An oligonucleotide which specifically binds to
Salmonella toxin gene invA mRNA at 41°C was selected.

(1) PCR was carried out on the region of base Nos.
104 to 2052 of the base sequence of Salmonella toxin gene
invA (Galan, J.E. et al., J. Bacteriol., <u>174</u>, 4338-4349
(1992), US GenBank Registered No. M 90846) using a
forward primer to which the promoter sequence of T7 RNA
polymerase was added at the 5' end and having a sequence
homologous to the above base Nos. 104 to 122, and a
reverse primer having a sequence complementary to the
above base Nos. 2029 to 2052.

(2) The above PCR product was used as a template in
preparing a standard RNA by a transcription reaction
employing T7 RNA polymerase (manufactured by Takara Shuzo

Co., Ltd.). The PCR-produced template was then digested
with a DNA polymerase (manufactured by Takara Shuzo Co.,
Ltd.), and the standard RNA was purified using CHROMA
SPIN™ 100 (manufactured by Toyobo Co., Ltd.).

5   (3) The standard RNA was quantified by ultraviolet
absorption at 260 nm, and then diluted to a concentration
of 0.45 pmol/μl with an RNA diluent (10 mM Tris-HCl (pH
8.0), 0.1 mM EDTA, 1mM DTT, 0.5 U/μl RNase Inhibitor).

           (4) A reaction solution in an amount of 9.0 μl
10   having the following composition was dispensed into 0.5
ml PCR tubes (Gene Amp Thin-Walled Reaction Tubes™,
manufactured by Perkin-Elmer Co., Ltd.).
           Reaction Solution Composition
           20.0 mM Tris-HCl buffer (pH 7.5)
15   20.0 mM potassium chloride
           10.0 mM magnesium chloride
           0.1 mM DTT
           0.1 mM EDTA
           0.9 μM standard RNA
20   2.0 μM oligonucleotide (oligonucleotide having any
of the sequences listed below being used)
           (Oligo-1): SEQ. ID. No. 1
           (Oligo-2): SEQ. ID. No. 2
           (Oligo-3): SEQ. ID. No. 3
25   (Oligo-4): SEQ. ID. No. 4
           (Oligo-5): SEQ. ID. No. 5
           (Oligo-6): SEQ. ID. No. 6
           (Oligo-7): SEQ. ID. No. 7
           (Oligo-8): SEQ. ID. No. 8
30   (Oligo-9): SEQ. ID. No. 9
           (Oligo-10): SEQ. ID. No. 10
           (Oligo-11): SEQ. ID. No. 11
           (Oligo-12): SEQ. ID. No. 12
           Distilled water for adjusting volume
35   (5) The reaction solutions were then incubated at

41°C for 5 minutes, and then 1 μl of 0.1 U/μl RNase H (manufactured by Takara Shuzo Co., Ltd.) was added thereto (RNase H: an enzyme that cleaves RNA of a double-stranded DNA/RNA).

5   (6)   Subsequently, the PCR tubes were incubated at 41°C for 15 minutes.

       (7)   Urea-modified polyacrylamide gel (acrylamide concentration: 6%; urea: 7 M) electrophoresis was conducted to confirm the cleaved fragments following the
10 reaction.   Dyeing following the electrophoresis was carried out with SYBR Green™ II (manufactured by Takara Shuzo Co., Ltd.).   Upon binding of the oligonucleotide to the specific sequence of the standard RNA (target RNA), RNA of the double stranded DNA/RNA was cleaved by RNase
15 H.   As a result, a characteristic band could be observed.

       The results of the electrophoresis are shown in Fig. 1.   When the oligonucleotide specifically binds to the standard RNA, the standard RNA will be digested in this binding region, yielding a digestion product having a
20 characteristic chain length.   Table 1 shows the position and expected band chain lengths resulting when the oligonucleotide specifically binds to the standard RNA. Cleavages at expected positions were confirmed for Oligo-1 to Oligo-12.   It was demonstrated that the
25 oligonucleotides firmly bind to the standard RNA, namely, Salmonella toxin gene invA mRNA, at 41°C and in a constant state.

Table 1

Oligonucleotide	Position[1]	Expected cleaved band lengths (base)
Oligo-1	225	225, 1724
Oligo-2	518	518, 1431
Oligo-3	569	569, 1380
Oligo-4	705	705, 1244
Oligo-5	742	742, 1207
Oligo-6	781	781, 1168
Oligo-7	881	881, 1068
Oligo-8	922	922, 1027
Oligo-9	955	955, 994
Oligo-10	985	985, 964
Oligo-11	1235	1235, 714
Oligo-12	1279	1279, 670

Note: 1) The position designates the 5' end number of the oligonucleotide which binds to the invA mRNA standard (1949 base).

Example 2

An RNA amplification reaction was carried out using an oligonucleotide probe which specifically binds to Salmonella toxin gene invA.

(1) The above Salmonella toxin gene invA mRNA was diluted to a concentration of 10^4 copies/5 µl with an RNA diluent (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 U/µl RNase Inhibitor (manufactured by Takara Shuzo Co., Ltd.), 5 mM DTT). In the control testing sections, only the diluent was used (Negative).

(2) A reaction solution in an amount of 20.8 µl having the following composition was dispensed into 0.5 ml PCR tubes (Gene Amp Thin-Walled Reaction Tubes™, manufactured by Perkin-Elmer Co., Ltd.). The above RNA sample in an amount of 5 µl was added.

Reaction Solution Composition (each concentration designating a concentration in a final reaction solution in an amount of 30 µl)

60 mM Tris-HCl buffer (pH 8.6)

13 mM magnesium chloride

90 mM potassium chloride

39 U RNase Inhibitor

1 mM DTT

0.25 mM of each of dATP, dCTP, dGTP and dTTP

3.6 mM ITP

3.0 mM of each of ATP, CTP, GTP and UTP

5   0.16 µM first oligonucleotide

1.0 µM second oligonucleotide

1.0 µM third oligonucleotide

13% DMSO

Distilled water for adjusting volume

10   (3) An RNA amplification reaction was carried out as explained below using oligonucleotides having a sequence listed in Table 2 as the first, second and third oligonucleotides.

(4) A solution was prepared in step (2) so that the

15   combination of the first, second and third oligonucleotides became one as listed in Table 2.

(5) After incubating the above reaction solution for 5 minutes at 41°C, 4.2 µl of an enzyme solution having the following composition was added.

20   Enzyme Solution Composition (each concentration designating a concentration in a final reaction solution in an amount of 30 µl)

1.7% sorbitol

3 µg bovine serum albumin

25   142 U T7 RNA polymerase (Gibco)

8 U AMV-Reverse Transcriptase (Takara Shuzo Co., Ltd.)

Distilled water for adjusting volume

(6) Subsequently the PCR tubes were incubated at

30   41°C for 30 minutes.

(7) In order to identify the RNA amplified portion following the reaction, agarose gel (agarose concentration of 4%) electrophoresis was performed. Dyeing following the electrophoresis was performed with

35   SYBR Green™ II (manufactured by Takara Shuzo Co., Ltd.).

When an oligonucleotide binds to the specific portion of the target RNA, the RNA portion between the second and third oligonucleotides is amplified, thereby enabling observation of a characteristic band.

5　　　　The results of the electrophoresis are shown in Fig. 2. The chain lengths of the specific bands amplified in the reaction are shown in Table 2. In the RNA amplification reactions using the combinations of oligonucleotides shown in Table 2, a specific band could

10　be confirmed in any of the combinations. Accordingly, it was demonstrated that these combinations are effective in detecting the target RNA.

Table 2

Combination	First oligonucleo-tide	Second oligonucleo-tide	Third oligonucleo-tide	Amplification product chain length (base)
(a)	2S	2F5	4R	206
(b)	2S	2F5	5R	243
(c)	3S	3F5	4R	155
(d)	3S	3F5	5R	192
(e)	5S	5F5	8R	199
(f)	2S	2F10	5R	206
(g)	3S	3F10	4R	155
(h)	3S	3F10	5R	192

15　　　　Table 2 shows the combinations of the first, second and third oligonucleotides used in the present example, as well as the chain lengths of the amplified specific bands resulting from the RNA amplification reactions using these combinations. The 3' end hydroxyl group of

20　each first oligonucleotide base sequence was aminated. In each second oligonucleotide base sequence, the region of the 1st "A" to the 22nd "A" from the 5' end corresponds to the T7 promoter region, and the subsequent region from the 23rd "G" to the 28th "A" corresponds to the enhancer

25　sequence.

First oligonucleotide

　　　　2S (SEQ. ID. No. 2)

　　　　3S (SEQ. ID. No. 3)

　　　　5S (SEQ. ID. No. 5)

30　Second oligonucleotide

2F5 (SEQ. ID. No. 19)

3F5 (SEQ. ID. No. 20)

5F5 (SEQ. ID. No. 21)

2F10 (SEQ. ID. No. 22)

5   3F10 (SEQ. ID. No. 23)

Third oligonucleotide

4R (SEQ. ID. No. 4)

5R (SEQ. ID. No. 5)

8R (SEQ. ID. No. 8)

10   Example 3

Detection in a various number of initial copies of Salmonella toxin gene invA mRNA was carried out using combinations of oligonucleotides according to the present invention.

15   (1) The same standard RNA as in Example 1 was diluted to concentrations ranging from 10^5 copies/5 μl to 10^2 copies/5 μl with an RNA diluent (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 U/μl RNase Inhibitor (manufactured by Takara Shuzo Co., Ltd.), 5mM DTT). In the control

20   testing sections, only the diluent was used (Negative).

(2) A reaction solution in an amount of 20.8 μl having the following composition was dispensed into 0.5 ml PCR tubes (Gene Amp Thin-Walled Reaction Tubes™, manufactured by Perkin-Elmer Co., Ltd.). The above RNA

25   sample in an amount of 5 μl was added.

Reaction Solution Composition (each concentration designating a concentration in a final reaction solution in an amount of 30 μl)

60 mM Tris-HCl buffer (pH 8.6)

30   17 mM magnesium chloride

90 mM potassium chloride

39 U RNase Inhibitor

1 mM DTT

0.25 mM of each of dATP, dCTP, dGTP and dTTP

35   3.6 mM of ITP

3.0 mM of each of ATP, CTP, GTP and UTP

0.16 μM first oligonucleotide (3S (SEQ. ID. No. 3) in Table 2, wherein its 3' end hydroxyl group is aminated)

1.0 μM second oligonucleotide (3F10 (SEQ. ID. No. 23) in Table 2)

1.0 μM third oligonucleotide (4R (SEQ. ID. No. 4) in Table 2)

25 nM intercalator fluorescent pigment-labeled oligonucleotide (SEQ. ID. No. 28, labeled with an intercalator fluorescent pigment at the phosphorus atom between the 13th "A" and the 14th "A" from the 5' end, and modified with a glycol group at its 3' end hydroxyl group)

13% DMSO

Distilled water for adjusting volume

(3) After incubating the above reaction solution for 5 minutes at 41°C, 4.2 μl of an enzyme solution having the following composition and pre-incubated for 2 minutes at 41°C was added.

Enzyme Solution Composition (each concentration designating a concentration in a final reaction solution in an amount of 30 μl)

1.7% sorbitol

3 μg bovine serum albumin

142 U T7 RNA polymerase (Gibco)

8 U AMV-Reverse Transcriptase (Takara Shuzo Co., Ltd.)

Distilled water for adjusting volume

(4) The PCR tube was then incubated at 41°C using a direct-measurable fluorescence spectrophotometer equipped with a temperature controller, and the reaction solution was periodically measured at an excitation wavelength of 470 nm and a fluorescent wavelength of 510 nm. Fig. 3 (upper panel) shows the time-course changes in the

fluorescence increase ratio (fluorescence intensity at predetermined time/background fluorescence intensity) of the sample, where enzyme was added at 0 minute. Fig. 3 (lower panel) shows the relationship between the logarithm of the initial RNA amount and the detection time (time at which the relative fluorescence reaches the negative sample's average value plus 3 standard deviations; i.e., the time to reach 1.2). The initial RNA amount was between 10^1 copies/30 µl and 10^5 copies/30 µl.

Fig. 3 shows that 10^2 copies were detected in about 13 minutes. A fluorescent profile and a calibration curve both depending on the initial concentration of the labeled RNA were obtained, indicating that it is possible to quantify the target RNA present in unknown samples. This demonstrates that rapid, highly sensitive detection of invA mRNA is possible by this method.

Example 4

An oligonucleotide which specifically binds to Salmonella toxin gene stn mRNA at 41°C was selected.

(1) PCR was carried out on the region of base Nos. 346 to 1092 of the base sequence of Salmonella toxin gene stn (Chopra, A. K. et al., Microb. Pathog., 16, 85-98 (1994), US GenBank Registered No. L 16014) using a forward primer to which the promoter sequence of T7 RNA polymerase was added at the 5' end having a sequence homologous to the above base Nos. 346 to 369, and a reverse primer having a sequence complementary to the above base Nos. 1076 to 1092.

(2) The above PCR product was used as a template for preparation of the standard RNA by a transcription reaction employing T7 RNA polymerase (manufactured by Takara Shuzo Co., Ltd.). The PCR-produced template was digested with a DNA polymerase (manufactured by Takara Shuzo Co., Ltd.), and the standard RNA was purified using CHROMA SPIN™ 100 (manufactured by Toyobo Co., Ltd.).

(3)   The standard RNA was quantified by ultraviolet absorption at 260 nm, and then diluted to a concentration of 0.45 pmol/μl with an RNA diluent (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1mM DTT, 0.5 U/μl RNase Inhibitor).

5   (4)   A reaction solution in an amount of 9.0 μl having the following composition was dispensed into PCR tubes (volume of 0.5 ml, Gene Amp Thin-Walled Reaction Tubes™, manufactured by Perkin-Elmer Co., Ltd.).
       Reaction Solution Composition

10   20.0 mM Tris-HCl buffer (pH 7.5)
       20.0 mM potassium chloride
       10.0 mM magnesium chloride
       0.1 mM DTT
       0.1 mM EDTA

15   0.9 μM standard RNA

       2.0 μM oligonucleotide (an oligonucleotide having any of the sequences listed below being used)
       (Oligo-13): SEQ. ID. No. 13
       (Oligo-14): SEQ. ID. No. 14

20   (Oligo-15): SEQ. ID. No. 15
       (Oligo-16): SEQ. ID. No. 16
       (Oligo-17): SEQ. ID. No. 17
       (Oligo-18): SEQ. ID. No. 18
       Distilled water for adjusting volume

25   (5)   The reaction solutions were then incubated at 41°C for 5 minutes, and then 1 μl of 0.1U/μl RNase H (manufactured by Takara Shuzo Co., Ltd.; an enzyme that cleaves RNA of a double-stranded DNA/RNA) was added thereto.

30   (6)   Subsequently, the PCR tubes were incubated at 41°C for 15 minutes.

       (7)   Urea-modified polyacrylamide gel (acrylamide concentration: 6%; urea: 7 M) electrophoresis was conducted to confirm the cleaved fragments following the

35   reaction.   Dyeing following the electrophoresis was

carried out with SYBR Green™ II (manufactured by Takara
Shuzo Co., Ltd.). Upon binding of the oligonucleotide to
the specific sequence of the standard RNA (target RNA),
RNA of the double stranded DNA/RNA is cleaved by RNase H.
As a result, a characteristic band could be observed.

The results of the electrophoresis are shown in Fig.
4. When the oligonucleotide specifically binds to the
standard RNA, the standard RNA will be digested in this
binding region, yielding a digestion product having a
characteristic chain length. Table 3 shows the position
and expected band chain lengths when an oligonucleotide
specifically binds to the standard RNA. Cleavages at
expected positions were confirmed for Oligo-13 to Oligo-
18. It was demonstrated that the oligonucleotides firmly
bind to Salmonella toxin gene stn mRNA at 41°C and in a
constant state.

Table 3

Oligonucleotide	Position[1]	Expected cleaved band lengths (base)
Oligo-13	59	59, 688
Oligo-14	191	191, 556
Oligo-15	311	311, 436
Oligo-16	421	421, 326
Oligo-17	642	642, 105
Oligo-18	671	671, 76

Note: 1) The position designates the 5' end number of an
oligonucleotide which binds to the stn mRNA standard (747 base).

Example 5

Combinations of oligonucleotides according to the
present invention were used for detection of different
initial copy numbers of Salmonella toxin gene stn mRNA.

(1) The same standard RNA of Salmonella toxin gene
stn mRNA as in Example 4 was diluted to a concentration
of 10^4 copies/5 μl with an RNA diluent (10 mM Tris-HCl
(pH 8.0), 1 mM EDTA, 0.5 U/μl RNase Inhibitor
(manufactured by Takara Shuzo Co., Ltd.), 5 mM DTT). In
the control testing sections, only the diluent was used
(Negative).

(2) A reaction solution in an amount of 20.8 µl having the following composition was dispensed into 0.5 ml PCR tubes (Gene Amp Thin-Walled Reaction Tubes™, manufactured by Perkin-Elmer Co., Ltd.). The above RNA sample in an amount of 5 µl was added.

Reaction Solution Composition (each concentration designating a concentration in a final reaction solution in an amount of 30 µl)

60 mM Tris-HCl buffer (pH 8.6)

17 mM magnesium chloride

90 mM potassium chloride

39 U RNase Inhibitor

1 mM DTT

0.25 mM of each of dATP, dCTP, dGTP and dTTP

3.6 mM ITP

3.0 mM of each of ATP, CTP, GTP and UTP

0.16 µM first oligonucleotide (combination in Table 4, hydroxyl group at the 3' end being aminated)

1.0 µM second oligonucleotide (combination in Table 4)

1.0 µM third oligonucleotide (combination in Table 4)

25 nM intercalator fluorescent pigment-labeled oligonucleotide (SEQ. ID. No. 29, labeled with an intercalator fluorescent pigment at the phosphorus atom between the 12th "A" and the 13th "A" from the 5' end, and modified with a glycol group at its 3' end hydroxyl group)

13% DMSO

Distilled water for adjusting volume

(3) After incubating the above reaction solution for 5 minutes at 41°C, 4.2 µl of an enzyme solution having the following composition and pre-incubated for 2 minutes at 41°C was added.

Enzyme Solution Composition (each concentration

designating a concentration in a final reaction solution in an amount of 30 μl)

1.7% sorbitol

3 μg bovine serum albumin

142 U T7 RNA polymerase (Gibco)

8 U AMV-Reverse Transcriptase (manufactured by Takara Shuzo Co., Ltd.)

Distilled water for adjusting volume

(4) The PCR tube was then incubated at 41°C using a direct-measurable fluorescence spectrophotometer equipped with a temperature controller, and the reaction solution was periodic measured at an excitation wavelength of 470 nm and a fluorescent wavelength of 510 nm.

Fig. 5 shows the time-course changes in the fluorescence increase ratio (fluorescence intensity at predetermined time/background fluorescence intensity) of the sample, where enzyme was added at 0 minute.

Defining the detection time as a time at which the relative fluorescence exceeds 1.2, Fig. 5 then shows that 10^4 copies can be detected in about 11 to 15 minutes. This demonstrates that rapid, highly sensitive detection of stn mRNA is possible by the method.

Table 4

Combination	First oligonucleotide	Second oligonucleotide	Third oligonucleotide	Amplification product chain length (base)
(i)	B1S	B1F5	B4R	233
(j)	B3S	B3F5	B4R	120
(k)	B1S	B1F10	B4R	233
(l)	B3S	B3F10	B4R	120

Table 4 shows the combinations of the first, second and third oligonucleotides used in the present example, as well as the chain lengths of the amplified characteristic bands resulting from the RNA amplification reactions using these combinations. The 3' end hydroxyl group of each first oligonucleotide base sequence was aminated. In each second oligonucleotide base sequence, the region of the 1st "A" to the 22nd "A" from the 5' end

corresponds to the T7 promoter region, and the subsequent region from the 23rd "G" to the 28th "A" corresponds to the enhancer sequence.

First oligonucleotide

5   B1S (SEQ. ID. No. 13)

          B3S (SEQ. ID. No. 14)

Second oligonucleotide

          B1F5 (SEQ. ID. No. 24)

          B3F5 (SEQ. ID. No. 25)

10   B1F10 (SEQ. ID. No. 26)

          B3F10 (SEQ. ID. No. 27)

Third oligonucleotide

          B4R (SEQ. ID. No. 15)

          Results

15   As explained above, the present invention provides oligonucleotides capable of complementarily binding to an intramolecular structure-free region of Salmonella toxin gene invA and stn mRNA, and a detection method using the oligonucleotides. Moreover, the present invention

20   provides oligonucleotides for detecting Salmonella toxin gene invA and stn mRNA, namely, oligonucleotide primers and oligonucleotide probes used for nucleic acid amplification methods. Because the oligonucleotides provided by the present invention are capable of

25   specifically binding to the target RNA at a relatively low and constant temperature, they are particularly suitable as primers used for a process of amplifying the target RNA.

          The amplification processes and detection methods

30   provided by the present invention use oligonucleotides favorable to the process of amplifying the target RNA as explained above. As a result, the present invention achieves the effect of making heat-denaturing of the target in advance unnecessary when the amplification

35   process is to be carried out at a relatively low and constant temperature.